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Evaluation of vaccines designed to induce protective cellular immunity against the *Plasmodium yoelii* circumsporozoite protein: vaccinia, pseudorabies, and salmonella transformed with circumsporozoite gene

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In an attempt to induce a protective cytotoxic T-cell mediated immunity against sporozoites of *Plasmodium yoelii*, the gene encoding the *P. yoelii* circumsporozoite (CS) protein was engineered into three live vectors, vaccinia, attenuated pseudorabies, and attenuated *Salmonella typhimurium*. *Balb/c* mice were immunized with 1–4 doses of 10^8 pfu of the vaccinia construct (IP), 3 doses of 10^5 , 10^6 or 10^7 pfu of pseudorabies construct (IV), and 3 doses of 10^9 salmonella transformants (orally). In the case of vaccinia and pseudorabies constructs, an excellent immune response was obtained as measured by antibodies to sporozoites. No protection or delay in prepatent period was seen in any of the experimental animals when challenged with 200 (vaccinia, pseudorabies) or 100 (salmonella) sporozoites, although mice immunized with irradiation-attenuated sporozoites were consistently protected against challenge with $>10^4$ sporozoites. Since other vaccinia, pseudorabies, and salmonella CS constructs have been shown to induce cytotoxic T lymphocytes (CTL) against the CS protein, it is likely that CTL against the CS protein were induced during these studies. It is currently unclear if the vaccines did not induce the appropriate CTL or inadequate numbers of CTL, or if CTL against the *P. yoelii* CS protein are inadequate to protect against sporozoite challenge.

Introduction

Both cellular (^{1–5}) and humoral immune mechanisms (^{3, 6, 7}) against the pre-erythrocytic stages of malaria have been shown to protect against sporozoite-induced malaria in rodent model systems. However, the potent, protective immunity, conferred by immunization with irradiation-attenuated sporozoites (^{8–11}) is dependent on CD8⁺ T cells and may be independent of antibody (^{1, 4, 5, 10}). Only live sporozoites are able to confer such immunity, and it is now thought that this is in part due to the requirement for production of the appropriate antigens within the hosts' cells so that they can be exported in the class I pathway, and presented to CD8⁺ T cells in combination with class I MHC molecules.

In an attempt to induce such immunity against the CS protein of *Plasmodium yoelii*, we have used vaccinia, pseudorabies, and *Salmonella typhimurium* transformed with the gene (¹²) encoding the *P. yoelii* CS protein as vaccines.

Materials and methods

Gene encoding the *P. yoelii* CS protein

The gene (¹²) encodes a protein of 391 amino acids (FACEP) (Fig. 1).

Construction of *P. yoelii* CS recombinants

Vaccinia *P. yoelii* CS recombinant (Vpy). Vpy and a control vaccinia construct containing the gene encoding an *Escherichia coli* galactokinase (Vgalk) were constructed as previously described (^{10, 13, 14}). Very briefly, a 1.3 Kb fragment encoding the entire *P. yoelii* CS protein coding region (Fig. 1) was isolated by PAGE and subcloned into the Sma I site of pGS20 such that the 7.5 Kb gene promoter directs transcription of the inserted DNA. Vaccinia virus recombinants were then generated by infecting monolayers of CV-1 cells with wild type vaccinia virus (TK⁺ Wyeth strain). Recombinant plasmid DNA was introduced by transfection of calcium phosphate precipitated DNA and the cells were harvested 48 h later. The virus was then released by three freeze-thaw cycles. TK⁺ recombinant virus selected from cell lysates was then screened for expression of the *P. yoelii* CS protein by immunoblot analysis. Finally,

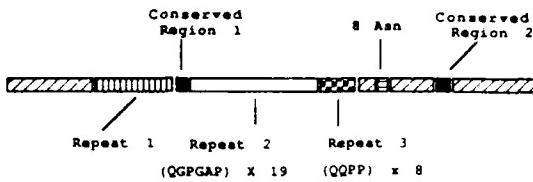
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Fig. 1. Schematic diagram of the gene encoding the *P. yoelii* CS protein.

plaque-purified virus was used to infect monolayers of CV-1 cells, and cell lysates were screened by Western blotting to confirm the presence of the full length *P. yoelii* CS protein.

Pseudorabies *P. yoelii* CS recombinant (PRV111). The pseudorabies virus was generated by inserting a chimeric gene into an attenuated parental virus. The thymidine kinase (TK) negative parental virus was obtained according to the method of Post & Roizman (15). The chimeric gene was constructed in a plasmid vector and utilizes the PRV gpX (glycoprotein X) promoter and polyadenylation signal (16) to express a hybrid coding region consisting of amino acids 1–269 of the PRV gpX gene followed in frame by amino acids 1–388 of the *P. yoelii* CS protein. This gene was excised from the plasmid construction using flanking *Xba*I sites, and then inserted directly into the two *Xba*I sites located in the parental virus DNA.

Transfection of the resulting recombinant molecules into Vero cells produced a virus containing two copies of the chimeric gene. PRV095 is a TK-negative virus similar to PRV111 which carries no foreign genes.

Salmonella *P. yoelii* CS recombinant (Sal/CS). A plasmid encoding the entire *P. yoelii* CS protein fused to 81 aminoacids of the non-structural protein of influenza A was used to transform LB 5000 (r^m). Ampicillin-resistant colonies were checked for the plasmid. Plasmid DNA was extracted from recombinants. An attenuated avirulent strain of *Salmonella typhimurium* (WR 4024 typr r^m) was transformed with DNA passed from LB 5000. Ampicillin-resistant colonies were checked for expression of the CS protein using NYS1, a monoclonal antibody that reacts with the *P. yoelii* CS protein. One transformant harbouring the plasmid and exhibiting a positive reaction with the monoclonal antibody was used to inoculate mice.

Sporozoites

The 17X (NL) strain of *P. yoelii* was used for all experiments. Sporozoites used for immunization were

isolated from infected mosquitos that had previously been irradiated with a dose of 10 Krads from a ¹³⁷Ce source. Discontinuous gradient centrifugation (17) was used to separate the sporozoites for immunization. Sporozoites used for challenging immunized mice were obtained by hand dissection of nonirradiated infected mosquitos.

Mice

Six- to 10-week old female BALB cByJ mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments.

Immunization and challenge schedule

(a) *Immunization with the vaccinia construct.* Mice were immunized by intraperitoneal (i.p.) injection of 10⁶ pfu/dose of Vpy, or the control vaccinia construct, Vgalk. A positive control group of mice immunized intravenously with irradiated sporozoites (5 × 10⁴ sporozoites first dose, and 3 × 10⁴ sporozoites for subsequent doses) was also included. Groups of 8 mice received 1–4 doses of either Vpy, Vgalk, or irradiated sporozoites at 2-week intervals. Two weeks after the last dose, blood for serum was obtained, the mice were challenged with infective sporozoites, and then monitored for parasitaemia for 14 days.

(b) *Immunization with the pseudorabies construct.* Mice were immunized i.v. with 10⁷, 10⁸, or 10⁹ pfu dose of the pseudorabies CS construct, PRV111. All doses were given 3 times at 3-week intervals. The control virus PRV095 was administered using the same schedule. Two weeks after the last dose, blood for serum was obtained, the mice were challenged with 200 infective sporozoites, and then monitored for 2 weeks for parasitaemia.

(c) *Immunization with the salmonella construct.* Fifteen mice were immunized by oral administration of 10⁹ transformed bacteria every other day for 5 days (total of 3 doses). Control mice received the untransformed vector. Four weeks after vaccination the mice were bled for serum, challenged with 100 sporozoites and subsequently monitored for parasitaemia.

Vaccines against *P. yoelii* circumsporozoite protein

Detection of antibodies to sporozoites

Indirect fluorescent antibody test (IFAT). Antibodies to air-dried sporozoites were detected in pooled sera by IFAT as previously described (7).

Enzyme linked immunosorbent assay (ELISA). ELISA was used to analyse the epitopes on the CS protein recognized by sera from the different groups of vaccinated mice. Briefly, the test consisted of a solid-phase assay in which synthetic peptides or recombinant proteins were coated to the wells of a 96-well flat-bottom microtitration plate. Wells were coated with one of the following: (a) PY CS.1, a recombinant fusion protein produced in *E. coli* that includes the three *P. yoelii* repeat domains and the conserved Region 1 sequence fused to 81 amino acids from the non-structural protein of influenza; (b) a synthetic peptide containing 3 repeating units of repeat #2, [QGPGAP]₃; and (c) a synthetic peptide containing 4 repeating units of repeat #3, [QQPP]₄. Each well was then blocked with casein and incubated overnight. The wells were then reacted with the appropriate dilution of a test serum, washed 3 times with buffer, after which the revealing antibody, a horse-radish peroxidase (HRP) goat anti-mouse Ig, was added followed by the peroxidase substrate consisting of equal volumes of H₂O₂ and ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline sulfonate]) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Colour development was measured spectrophotometrically at 405 nm using a Microelisa autoreader (MR 580) (Dynatech Laboratories, Alexandria, VA).

Results

Immunogenicity and protective efficacy of vaccines

Vaccinia construct (Vpy). Four doses of Vpy did not protect against challenge with 200 sporozoites, while 2 doses of irradiated sporozoites protected 100% of mice against challenge with 10⁴ sporozoites (Table 1). Although the aim of this study was to induce a protective cellular response, antibody levels to sporozoites were measured to determine if Vpy presented CS protein to the immune system. The results of IFAT (Table 1) and ELISA (Fig. 2 and 3) indicate that CS protein was expressed. Interestingly, although irradiated sporozoites induce an antibody response primarily directed against (QGPGAP)₂, antibodies induced by Vpy were higher against the recombinant fusion protein produced in *E. coli*, PY CS.1, and the minor repeat (QQPP)₃.

Pseudorabies construct (PRV111). None of the mice immunized with three doses of PRV111 were protected against challenge with 200 sporozoites. Anti-sporozoite antibody titre, as measured by IFAT, were comparable in the groups that were vaccinated with irradiated sporozoites and those immunized with PRV111 (Table 2), indicating that the CS protein was produced in immunized animals.

When the sera were tested in ELISA, the results were similar to those found after immunization with the vaccinia construct. Sera from mice immunized with irradiated sporozoites had higher levels of anti-

Table 1. Results of challenge of mice immunized with the vaccinia CS gene construct (Vpy), the control vaccinia recombinant (Vgalk), irradiated sporozoites (IRRspz) and of normal mice (Control)

No. of doses of vaccine	Immunogen	IFAT titre	No. protected/ No. challenged
<i>Challenged with a high dose (10000 sporozoites/mouse)</i>			
1	Vpy	32	0/8
	Vgalk	<8	0/8
	IRRspz	16	3/8
	Control	<8	0/8
2	Vpy	512	0/8
	Vgalk	<8	0/8
	IRRspz	2 ¹⁰	8/8
	Control	<8	0/8
3	Vpy	2048	0/8
	Vgalk	<8	0/8
	IRRspz	2 ¹⁰	5/3
	Control	<8	0/8
<i>Challenged with a low dose (200 sporozoites/mouse)</i>			
4	Vpy	1024	0/8
	Vgalk	<8	0/8
	IRRspz	2048	8/8
	Control	<8	0/8

Fig. 2. ELISA determination of the antibody response to the Hinc II fusion peptide of mice immunized with vaccine preparations IRRspz, Vpy and Vgalk.

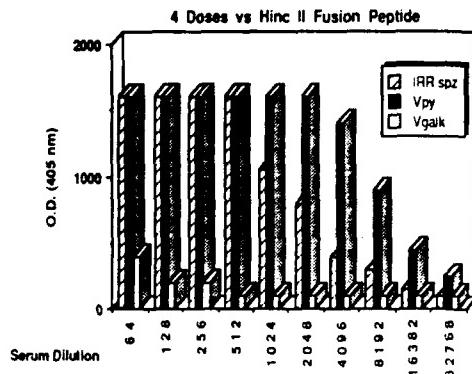
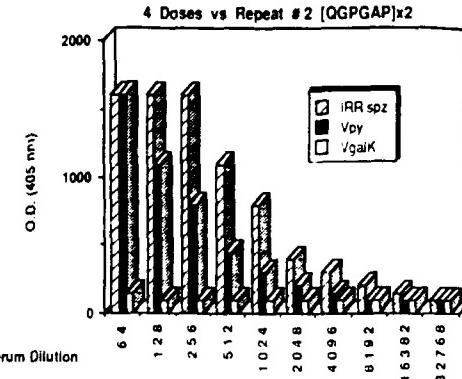


Fig. 3. ELISA determination of the antibody response to the synthetic repeat #2 (QGPGAP)₂ peptide of mice immunized with vaccine preparations IRRspz, Vpy and Vgalk.



bodies to the major repeat (QGPGAP)₂, while sera from the mice immunized with PRV111 had higher levels of antibodies to PY CS1, the recombinant fusion protein including 66% of the *P. yoelii* CS protein.

Vaccination with salmonella construct. None of the mice were protected and, as expected (18), none produced antibodies to CS protein (Table 3).

Discussion

The aim of this study was to induce protective cellular immunity against the *P. yoelii* CS protein by immunizing with vaccines that would produce the CS protein within the hosts' cells. Failure to protect mice with the vaccinia, pseudorabies, and *Salmonella typhimurium* constructs suggests that immunization with these constructs did not induce cytotoxic T cells against the CS protein and did not induce adequate numbers of CTL

Table 2. Results of challenge of mice immunized with the pseudorabies CS gene construct (PRV111), the control pseudorabies virus product (PRV095), irradiated sporozoites (IRRspz), and of normal mice (Control); all mice were challenged with 200 sporozoites

Experiment	Doses	Groups	iFAT titre	No. protected/ No. challenged
A	3	10^7 PRV111	1:4096	0:9
B	3	10^8 PRV111	1:1024	0:9
C	3	10^3 PRV111	1:128	0:9
D	3	10^7 PRV095	1:8	0:9
E	3	IRRspz	1:2048	0:9
F		Control	1:8	0:9

Table 3. Results of challenge of mice immunized with the salmonella CS gene construct (Sal/CS), the control salmonella product (Sal Control), and of normal mice (Control); all mice were challenged with 100 sporozoites

Immunogen	No. of mice	Treatment	No. infected/ No. challenged
Sal/CS	15	Three oral doses of 10^7 units of Sal/CS	15/15
Sal Control	15	Three oral doses of 10^8 units of Sal	15/15
Control	10	No treatment, infectivity control	10/10

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against the CS protein, or that CTL directed against the *P. yoelii* CS protein cannot protect against sporozoite-induced *P. yoelii* infections.

In these studies we did not determine if the vaccines induced CTL against the CS protein. However, there is considerable evidence suggesting that they did. Immunization with vaccinia containing the *P. falciparum* CS protein (19) and salmonella transformed with the *P. berghei* CS gene (Aggarwal, personal communication) and another salmonella transformed with *P. yoelii* CS protein (Weiss, personal communication) have been shown to induce CTL against the CS protein. Our data using another pseudorabies construct (Sedegah, personal observation) indicate that pseudorabies transformed with the *P. yoelii* CS gene induces CTL against the CS protein.

If CTL had been produced, then it is possible that the magnitude of the immune response was not adequate to prevent infection. Sadoff et al. (18) were able to protect mice against *P. berghei* infection by immunizing with a salmonella CS construct prepared in a similar manner to the *P. yoelii* CS construct used in this experiment. The reason for the differences in the protective immunity observed after immunization with these two vaccines may be a reflection of the infectivity of *P. yoelii* and *P. berghei* sporozoites. In the experiment with the salmonella transformant described in this paper the infectious dose required to infect 50% of mice (ID₅₀) was approximately 1.6 sporozoites. We therefore infected with approximately 100 ID₅₀s. In the *P. berghei* system the ID₅₀ is generally 200–500 sporozoites. Sadoff et al. (18) were able to protect against challenge with 1500 sporozoites, or approximately 3 to 10 ID₅₀s. It is quite likely that the magnitude of the CTL response was inadequate to protect against such an enormous infectious challenge in the *P. yoelii* system.

The aim of this work was to induce protective CTL against the CS protein. However, immunization with two of the constructs, Vpy and PRV111, was accompanied by the production of high levels of antibodies. When mice are immunized with irradiated *P. yoelii* sporozoites, antibodies alone do not protect against *P. yoelii* sporozoite challenge (5, 10) and the antibodies induced by immunization with the vaccinia and pseudorabies constructs were likewise not protective. Interestingly, immunization with irradiated sporozoites primarily induced antibodies to (QPGPAP)₂, the major repeat, while immunization with the subunit vaccines primarily induced antibodies to other regions of the CS protein.

In summary, vaccinia, pseudorabies, and salmonella constructs that contained the entire gene encoding the *P. yoelii* CS protein were used as vaccines to produce the CS protein within host cells. All three failed to protect mice against challenge. Work is

underway to determine if immunization with these vaccines did or did not induce CTL against the CS protein and, if so, to develop methods for improving this immune response.

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